RECOMBINANT HUMAN ALBUMIN FUSION PROTEINS WITH LONG-LASTING BIOLOGICAL EFFECTS

Inventors: Zailin Yu and Yan Fu

BACKGROUND OF THE INVENTION

Cross Reference to Related Application

This application claims the priority benefit of U.S. Provisional Application Serial No: 60/392,948 filed July 1, 2002, which is hereby incorporated herein by reference in its entirety.

Field of the Invention

This invention relates to the manufacture and use of recombinant albumin fusion proteins and combinations thereof, and particularly to yeast expressed fusion proteins formed between human albumin and bioactive molecules such as therapeutic proteins and peptides, and more particularly to yeast expressed fusion proteins formed between human albumin and cell proliferation stimulatory factor (CPSF), such as, blood cell-stimulatory factors, erythropoietin (EPO), interleukins (ILs), stem cell factor (SCF), thrombopoietin (TPO), granulocyte colony stimulating factor (G-CSF), and granulocyte macrophage colony stimulating factor (GM-CSF).

Description of Related Art

1. Albumin

Albumin is a soluble, monomeric protein which comprises about one-half of the blood serum protein. Albumin functions primarily as a carrier protein for steroids, fatty acids, and thyroid hormones and plays a role in stabilizing extracellular fluid volume. Mutations in this gene on chromosome 4 result in various anomalous proteins. Albumin is a globular un-glycosylated serum protein of molecular weight 65,000. The human

albumin gene is 16,961 nucleotides long from the putative 'cap' site to the first poly(A) addition site. It is split into 15 exons which are symmetrically placed within the 3 domains that are thought to have arisen by triplication of a single primordial domain. Albumin is synthesized in the liver as pre-pro-albumin which has an N-terminal peptide that is removed before the nascent protein is released from the rough endoplasmic reticulum. The product, proalbumin, is in turn cleaved in the Golgi vesicles to produce the secreted albumin. HSA has 35 cysteins; in blood this protein monomer has 17 disulfide linkage (Brown, J. R. "Albumin structure, Function, and Uses" Pergamon, New York, 1977). HSA is misfolded when produced intracellularly in yeast without its amino terminal secretion peptide sequence. This conclusion is based on its insolubility, loss of great than 90% of its antigenicity (as compared to human-derived HSA), and formation of large protein aggregates. At present albumin for clinical use is produced by extraction from human blood. The production of recombinant albumin in microorganisms has been disclosed in EP 330 451 and EP 361 991.

Albumin is a stable plasma transporter function provided by any albumin variant and in particular by human albumin. HSA is highly polymorphic and more than 30 different genetic alleles have been reported (Weikamp L, R, et al., Ann. Hum. Genet., 37 219-226, 1973). The albumin molecule, whose three-dimensional structure has been characterized by X-ray diffraction (Carter D.C. et al., Science 244, 1195-1198, 1989), was chosen to provide the stable transporter function because it is the most abundant plasma protein (40g per liter in human), it has a high plasma half-life (14-20 days in human, Waldmann T. A., in "Albumin Structure, Function and Uses", Rosenoer V. M. et al (eds), Pergamon Press, Oxford, 255-275,1977), and above all it has the advantage of being devoid of enzymatic function, thus permitting its therapeutic utilization at high dose.

2. Interleukin-11 (IL-11)

Human IL-11 (Paul et al. (1990), Pro. Natl. Acad. Sci. 87:7512) has been identified in medium conditioned by primate bone marrow-derived stromal cells. IL-11 is expressed in cells of mesenchymal origin, such as stromal fibroblasts, fetal lung fibroblasts and trophoblasts.

IL-11, also called adipogenesis inhibitory factor (AGIF), acts on hematopoietic progenitor cells and stromal cells (Kawashima, I., et al., Progress in Growth Factor Research, 4,191 1992). The mature molecule is a non-glycosylation protein, 178aa in length, and has an apparent molecular weight 23KD (as determined by SDS-PAGE). Human IL-11 gene consists of five exons and four introns and was mapped on chromosome 19 at band 19q13.3-q13.4. IL-11 exhibits a primary structure unrelated to that of known cytokines, but it often acts similar to other cytokines, notably IL-6. IL-11 is a pleiotropic growth factor effecting hematopoietic and non-hematopoietic cells, often in synergy with interleukins, colony stimulating factors, or stem cell factor. In hematopoietic cells, IL-11 can enhance megakaryopoiesis, stimulate early and intermediate myeloid progenitor cells, initiate proliferation of dormant hematopoietic progenitor cells, and stimulate T-cell-dependent development of antibody-secreting B-cells. In non-hematopoietic cells, IL-11 can inhibit adipogenesis, and mediates the hepatic acute phase response. IL-11 stimulated the production of erythrocytes was reported only by Quesniaux, VFJ., et al., Blood, 80, 1218 (1992).

Human IL-11 (e.g., NEURMEGA®, manufactured by America Home Products Company) has been approved for clinical trials in the United States for directly stimulating the proliferation of hematopoietic stem cells and megakaryocyte progenitor cells and inducing megakaryocyte maturation, resulting in increased platelet production. It has been used for the prevention of severe thrombocytopenia and the reduction of the need for platelet transfusion following myelosuppressive chemotherapy.

3. Erythropoietin (EPO)

Erythropoietin (EPO) is a glycoprotein that is the principle regulator of red blood cells growth and differentiation (US Patent No: 5,547,933). Erythropoiesis, the production of red blood cells, occurs continuously throughout the human life span to offset cell destruction. Erythropoiesis is a very precisely controlled physiological mechanism enabling sufficient numbers of red blood cells to be available in the blood for proper tissue oxygenation, but not so many that the cells would impede circulation. The formation of red blood cells occurs in the bone marrow and is under the control of the hormone EPO.

EPO is an acidic glycoprotein (~30,400 Daltons) produced primarily by the kidney and is the principal factor regulating red blood cell production in mammals. Renal production of EPO is regulated by changes in oxygen availability. Under conditions of hypoxia, the level of EPO in the circulation increases and this leads to increased production of red blood cells. The over-expression of EPO may be associated with certain pathophysiological conditions. Polycythemia exists when there is an overproduction of red blood cells (RBCs). Primary polycythemias, such as Polycythemia vera, are caused by EPO-independent growth of erythrocytic progenitors from abnormal stem cells and low to normal levels of EPO are found in the serum of affected patients.

On the other hand, various types of secondary polycythemias are associated with the production of higher than normal levels of EPO. The overproduction of EPO may be an adaptive response associated with conditions that produce tissue hypoxia, such as living at high altitude, chronic obstructive pulmonary disease, cyanotic heart disease, sleep apnea, high-affinity hemoglobinopathy, smoking, or localized renal hypoxia. In other instances, excessive EPO levels are the result of production by neoplastic cells. Cases of increased EPO production and erythrocytosis have been recorded for patients with renal carcinomas, benign renal tumors, Wilms' tumors, hepatomas liver carcinomas, cerebellar hemangioblastomas, adrenal gland tumors, smooth muscle tumors, and leiomyomas.

Deficient EPO production is found in conjunction with certain forms of anemias. These include anemia of renal failure and end-stage renal disease, anemias of chronic disorders [chronic infections, autoimmune diseases, rheumatoid arthritis, AIDS, malignancies], anemia of prematurity, anemia of hypothyroidism, and anemia of malnutrition. Many of these conditions are associated with the generation of IL-1 and TNF- , factors that have been shown to be inhibitors of EPO activity. Other forms of anemias, on the other hand, are due to EPO-independent causes and affected individuals show elevated levels of EPO. These forms include aplastic anemias, iron deficiency anemias, thalassemias, megaloblastic anemias, pure red cell aplasias, and myelodysplastic syndromes.

The amount of erythropoietin in the circulation is increased under conditions of hypoxia when oxygen transport by blood cells in the circulation is reduced. Hypoxia may

be caused by loss of large amounts of blood through hemorrhage, destruction of red blood cells by over-exposure to radiation, reduction in oxygen intake due to high altitudes or prolonged unconsciousness, or various forms of anemia. In response to tissues undergoing hypoxic stress, erythropoietin will increase red blood cell production by stimulating the conversion of primitive precursor cells in the bone marrow into proerythroblasts which subsequently mature, synthesize hemoglobin and are released into the circulation as red blood cells. When the number of red blood cells in circulation is greater than needed for normal tissue oxygen requirements, erythropoietin in circulation is decreased.

EPO may occur in three forms: alpha-, beta and asialo-EPO. The alpha- and beta-forms differ slightly in carbohydrate components, but have the same potency, biological activity and molecular weight. The asialo-form is an alpha- or beta-form with the terminal carbohydrate (sialic acid) removed. EPO is present in very low concentrations in plasma when the body is in a healthy state wherein tissues receive sufficient oxygenation from the existing number of erythrocytes. This normal low concentration is enough to stimulate replacement of red blood cells which are lost normally through aging. See generally for references, Testa, et al., Exp. Hematol., 8(Supp. 8), 144-152 (1980); Tong, et al., J.Biol.Chem., 256(24), 12666-12672 (1981); Goldwasser, J.Cell.Physiol., 110(Supp 1), 133-135 (1982); Finch, Blood, 60(6), 1241-1246 (1982); Sytowski, et al., Exp.Hematol., 8(Supp 8), 52-64 (1980): Naughton, Ann.Clin.Lab.Sci., 13(5), 432-438 (1983); Weiss, et al., Am.J.Vet.Res., 44(10), 1832-1835 (1983); Lappin, et al., Exp.Hematol., 11(7), 661-666 (1983); Baciu, et al., Ann.N.Y.Acad. Sci., 414, 66-72 (1983); Murphy, et al., Acta.Haematologica Japonica, 46(7), 1380-1396 (1983); Dessypris, et al., Brit.J.Haematol , 56, 295-306 (1984) ; and, Emmanouel, et al., Am.J.Physiol., 247 (1 Pt 2), F168-76 (1984).

Because EPO is essential in the process of red blood cell formation, the hormone has potential useful application in both the diagnosis and the treatment of blood disorders characterized by low or defective red blood cell production. See, generally, Pennathur-Das, et al., Blood, 63(5), 1168-71 (1984) and Haddy, Am.Jour.Ped.Hematol./Oncol., 4, 191-196, (1982) relating to erythropoietin in possible therapies for sickle cell disease, and Eschbach, et al. J.Clin.Invest., 74(2), pp. 434-441, (1984), describing a therapeutic

regimen for uremic sheep based on in vivo response to erythropoietin-rich plasma infusions and proposing a dosage of 10 U EPO/kg per day for 15-40 days as corrective of anemia of the type associated with chronic renal failure. See also, Krane, Henry Ford Hosp.Med.J., 31(3), 177-181 (1983).

Prior attempts to obtain erythropoietin in good yield from plasma or urine have proven relatively unsuccessful. Complicated and sophisticated laboratory techniques are necessary and generally result in the collection of very small amounts of impure and unstable extracts containing erythropoietin. Genetically engineered EPO (US Patent, 5,547,933) has been expressed in Chinese Hamster Ovary cell line (CHO). It has been approved for administration on clinical trials by FDA and now manufactures by Amgen Inc. under the name of EPOGEN.

It has been estimated that the availability of erythropoietin in quantity would allow for treatment each year of anemias of 1,600,000 persons in the United States alone. See, e.g., Morrison, "Bioprocessing in Space--an Overview", pp. 557-571 in The World Biotech REPOrt 1984, Volume 2:USA, (Online Publications, New York, N.Y. 1984). Recent studies have provided a basis for projection of efficacy of erythropoietin therapy in a variety of disease states, disorders and states of hematologic irregularity: Vedovato, et al., Acta.Haematol, 71, 211-213 (1984) (beta-thalassemia); Vichinsky, et al., J.Pediatr., 105(1), 15-21 (1984) (cystic fibrosis); Cotes, et al., Brit.J.Obstet.Gyneacol., 90(4), 304-311 (1983) (pregnancy, menstrual disorders); Haga, et al., Acta.Pediatr.Scand., 72, 827-831 (1983) (early anemia of prematurity); Claus-Walker, et al., Arch.Phys.Med.Rehabil., 65, 370-374 (1984) (spinal cord injury); Dunn, et al., Eur.J.Appl.Physiol., 52, 178-182 (1984) (space flight); Miller, et al., Brit.J.Haematol., 52, 545-590 (1982) (acute blood loss); Udupa, et al., J.Lab.Clin.Med., 103(4), 574-580 and 581-588 (1984); and Lipschitz, et al., Blood, 63(3), 502-509 (1983) (aging); and Dainiak, et al., Cancer, 51(6), 1101-1106 (1983) and Schwartz, et al., Otolaryngol., 109, 269-272 (1983) (various neoplastic disease states accompanied by abnormal erythropoiesis).

4. Granulocyte Colony Stimulating Factor (G-CSF)

Granulocyte colony stimulating factor (G-CSF) is produced by monocytes and fibroblasts. It stimulating granulocyte colony formation, activates neutrophils,

differentiates certain myeloid leukemic cell lines and is a potent activator of mature granulocytes (Metcalf D., cell, 43, 5, 1985; Groopman, J. E., Cell,. 50, 5 1987);. Nature human G-CSF is a 19.6 KD glycoprotein having 177 amino acids (Souza, L.M et al., Science, 232, 62, 1986). Human and murine GCSF share approximately 75% amino acid sequence homology and have biology cross-reactivity (Morstyn, G., and Burgess, A., Cancer Res., 48, 5624, 1988). The biological activity of recombinant human G-CSF was measured in a cell proliferation assay using NFS-60 cells (Shurafuji, N et al., Exp. Hematol., 17, 116, 1989). Human G-CSF has been brought to the market under the name of NEUPOGEN® by Amgen, Inc.

5. Granulocyte-Macrophage colony stimulating factor (GM-CSF)

Granulocyte-Macrophage colony stimulating factor (GM-CSF) induces myeloid progenitor cells from bone marrow to from colonies contains macrophages and granulocytes in semisolid media. GM-CSF also acts upon mature macrophages, eosinophils and nutrophils to stimulate various functional activities (Mazur, E., and Cohen, J., Clin Pharmacol. Ther., 46, 250, 1989; Morstyn, TG., and Burgess. A., Cancer Res., 48, 5624, 1988). GM-CSF is an acidic glycoprotein {18-22 KD human (Wong, G., et al., Science, 228, 810, 1986), 23 KD mouse (Metcalf, D., Blood, 67, 257, 1986)} which binds to high affinity receptors on GM-CSF sensitive cells. Although human and mouse GMCSF share 54% amino acid sequence homology, their biological actions are species-specific (Metcalf, D., Blood, 67, 257, 1986). Other growth factors and CSFs modulate receptor binding or actions of GM-CSF (Nicola, N., Immunol. Today, 8, 134, 1987). The proliferative activity of human GMCSF is tested in culture using human TF-1 cells (Kitamura, T., et al., J. cell Physiol., 140, 323, 1989). Human GM-CSF has been brought to the market under the name of LEUKINE® by Immunex, Inc

6. Macrophage Colony Stimulating Factor (M-CSF)

Macrophage Colony Stimulating Factor (M-CSF) is produced by monocytes, fibroblasts and endothelial cells. It stimulates the formation of macrophage colonies (Metcalf, D., Blood, 67, 257, 1986), enhances antibody-dependent cell mediated cytotoxicity by monocytes and macrophages (Mufson, R. A. et al., Cellular Immunol.,

119, 182, 1989), and inhibits bone resorption by osteoclasts (Hattersley, G., et al., J. Cell Physiol., 137, 199, 1988). M-CSF is glycoprotein and appears in a few different molecular weight forms due to variation in glycosylation. The peptide has 159 amino acids (Kawasaki, E. S., et al., Science, 230, 291, 1985).

7. Thrombopoietin (TPO)

Thrombopoietin (TPO), the ligand for the receptor encoded by the c-Mpl proto-oncogene, acts as a stimulator of the development of megakaryocyte precursors of platelets. Similar to erythropoietin, TPO leads to an increase in number of circulating platelets. TPO affects the entire thrombopoietic process, with stronger effects in the later stages. Other thrombopoietic cytokines include Stem cell factor (SCF), IL-3, IL-6 and IL-11.

TPO is an approximately 35KD polypeptide of 335 amino acid. However, due to glycosylation the protein has an apparent =mol weight of 75KD in SDS-PAGE. The precursor form of TPO consists of 356 amino acids. To generate the mature TPO (335aa), the precursor cleaves a 21 amino acids signal peptide. Human, mouse and dog TPO shows 69-75% amino acid homology. The biological activities of recombinant human TPO was measured in a cell proliferation assay using MO7e cells.

8. *Interleukin-3 (IL-3)*

Interleukin -3 (IL-3) is one of a large and growing group of growth factors which support the proliferation and differentiation of hematopoietic progenitors as well as cells committed to various myeloid lineages in vitro and in vivo. Human IL-3 has 133 amino acids in mature protein and the glycosylation is not necessary for biological activity in vitro and in vivo. The homology between human and murine IL-3 is considerably less. The initial studies on the biology and biochemistry of IL-3 shows that among the well characterized hematopoietic growth factors, IL-3, is the only factor to be predominantly, if not exclusively, produced by activated T cell in normal cells in mice (Ihele and Weinstein, 1986) as well as in human (Yang and Clark 1998). The structure of IL-3, and the structure and location of its gene, are very much like those of a number of the hematopoietic growth factors and suggest that IL-3 is a member of an evolutionarily

related family of growth factors. In preclinical and clinical trials, the most prominent and consistent effect of Il-3 in vivo is a significant increase in the absolute neutrophil count (ANC). In vitro IL-3, in combination with other cytokines such as stem-cell factor, IL-6, IL-1, IL-11, G-CSF. GM-CSF, erythropoietin (EPO), or Thrombopoietin (TPO) induces the proliferation of colony-forming units granulocyte-macrophage (CFU-GM), CFU-Eo, CFU-Baso, burst-forming units-erythroid (BFU-E), colony-forming units-megakaryocyte (CFU-MK) and colony-forming units-granulocyte/erythroid/macrophage/megakaryocyte (CFU-GEMM) in semisolid medium, and it stimulates the proliferation of purified CD34+ cells in suspension culture (Eder, et al., Stem Cell, 15:327-333, 1997).

SUMMARY OF THE INVENTION

The present invention provides compositions, kits and methods for promoting general health or for prevention or treatment of diseases by using novel recombinant fusion proteins of human serum albumin (HSA) and bioactive molecules. The bioactive molecules may be a protein or peptide having a biological function in vitro or in vivo, and preferably, having a therapeutic activity when administered to a human. It is believed that by fusing the bioactive molecule to HSA, stability of the bioactive molecule in vivo may be improved and the therapeutic index increased due to reduced toxicity.

In one aspect of the invention, recombinant fusion proteins of human serum albumin (HSA) and a cell proliferation stimulatory factor (CPSF) are provided 1) to stimulate proliferation of multiple cell types, especially cells of various developmental lineages in the blood, 2) allow a slower release of the HSA-CPSF fusion in the body to maximize the therapeutic effects of the CPSF, and/or 3) to reduce potential side effects or toxicity associated with administration of CPSF alone. In addition, manufacturing processes are provided for efficient, cost-effective production for producing these recombinant proteins in yeast.

In another aspect of the invention, an isolated polynucleotide is provided that encodes a fusion protein formed between HSA and a CPSF, i.e., an HSA/CPSF fusion. The CPSF may include any protein that can stimulate cell proliferation and/or production, preferably selected from the group consisting of colony-stimulatory factors such as colony-stimulating factors such as G-CSF, GM-CSF, eosinophil (EOS)-CSF (i.e. Interleukin-5), macrophage (M)-CSF (CSF-1), multi-CSF(i.e. IL-3) and erythropoietin (EPO); interleukins such as IL-1; IL-2; IL-4; IL-6; IL-7; IL-9; IL-10; IL-11; IL-12; IL-13 and IL-18; Steel factors (SLF: c-kit ligand; Stem-cell factor (SCF); mast cell growth factor); erythroid potentiating activity (EPA), Lactoferrin (LF), H-subunit ferritin (i.e., acidic isoferritin), prostaglandin (PG) E1 and E2, tumor necrosis factor (TNF)-α, -β (i.e. lymphotoxin), interferon (IFN) –α (1b, 2a and 2b), -β, -ω and -γ; transforming growth factor (TGF)-β, activin, inhibin, leukemic inhibitory factor, oncostatin M; and chemokines such as macrophage inflammatory protein (MIP)-1-α (i.e. Stem-cell

inhibitor); macrophage inflammatory protein (MIP)-1β; macrophage inflammatory protein (MIP)-2-α (i.e., GRO-β); GRO-α; MIP-2-β (i.e., GRO-γ); platelet factor-4; IL-8; macrophage chemotactic and activating factor and IP-10.

The CPSF may be linked directly to the N-terminus or the C-terminus of HSA to form an HSA-CPSF fusion. Optionally, there is a peptide linker (L) linking HSA and CPSF together to form the fusion protein: HSA-L-CPSF, or CPSF-L-HSA. The length of peptide is preferably between 2-100 aa, more preferably between 5-50 aa, and most preferably between 14-30 aa. The peptide linker may be a flexible linker that minimizes steric hindrance imposed by the bulky HSA protein on CPSF, such as a $(G_4S)_{3-4}$ linker.

In one embodiment, an isolated polynucleotide is provided that encodes a human serum albumin-interleukin-11 fusion protein (HSA-IL-11). The polynucleotide comprises a nucleotide sequence at least 90% identical to SEQ ID NO. 1 (**Figure 1**). Preferably, the polynucleotide comprises a nucleotide sequence at least 95% identical to SEQ ID NO. 1. Preferably, the polynucleotide encodes an amino acid sequence comprising SEQ ID NO. 2.

In one embodiment, an isolated polynucleotide is provided that encodes a human serum albumin-interleukin-3 fusion protein (HSA-IL-3). The polynucleotide comprises a nucleotide sequence at least 90% identical to SEQ ID NO. 3. Preferably, the polynucleotide comprises a nucleotide sequence at least 95% identical to SEQ ID NO. 3. Preferably, the polynucleotide encodes an amino acid sequence comprising SEQ ID NO. 4.

In another embodiment, an isolated polynucleotide is provided that encodes a human serum albumin-erythropoietin fusion protein (HSA-EPO). The polynucleotide comprises a nucleotide sequence at least 90% identical to SEQ ID NO. 5. Preferably, the polynucleotide comprises a nucleotide sequence at least 95% identical to SEQ ID NO. 5. Preferably, the polynucleotide encodes an amino acid sequence comprising SEQ ID NO. 6. [HSA-EPO].

In yet another embodiment, an isolated polynucleotide is provided that encodes a human serum albumin-granulocyte colony stimulating factor fusion protein (HSA-GCSF). The polynucleotide comprises a nucleotide sequence at least 90% identical to

SEQ ID NO.7. Preferably, the polynucleotide comprises a nucleotide sequence at least 95% identical to SEQ ID NO. 7. Preferably, the polynucleotide encodes an amino acid sequence comprising SEQ ID NO. 8.

In yet another embodiment, an isolated polynucleotide is provided that encodes a human serum albumin-granulocyte macrophage colony stimulating factor fusion protein (HSA-GMCSF). The polynucleotide comprises a nucleotide sequence at least 90% identical to SEQ ID NO. 9. Preferably, the polynucleotide comprises a nucleotide sequence at least 95% identical to SEQ ID NO. 9. Preferably, the polynucleotide encodes an amino acid sequence comprising SEQ ID NO. 10.

In yet another embodiment, an isolated polynucleotide is provided that encodes a human serum albumin-CPSF fusion protein (HSA-CPSF). The polynucleotide comprises a nucleotide sequence at least 90% identical to SEQ ID NO. 11. Preferably, the polynucleotide comprises a nucleotide sequence at least 95% identical to SEQ ID NO. 11. Preferably, the polynucleotide encodes an amino acid sequence comprising SEQ ID NO. 12. Optionally, the polynucleotide further comprises a nucleotide sequence at least 90% identical to SEQ ID NOs. 13, 15, 17, 19, or 21. Preferably, the polynucleotide further comprises a nucleotide sequence encoding an amino acid sequence comprising SEQ ID NOs. 14, 16, 18, 20, or 22.

According to the embodiment, the CPSF may be selected from the group consisting of G-CSF, GM-CSF, (EOS)-CSF, CSF-1, EPO, IL-1; IL-2, IL-3, IL-4; IL-6; IL-7; IL-8, IL-9; IL-10; IL-11; IL-12; IL-13, IL-18, SLF, SCF, mast cell growth factor, EPA, Lactoferrin, H-subunit ferritin, prostaglandin (PG) E1 and E2, TNF-α, TNF-β, IFN–α, IFN-β, IFN-ω, IFN -γ; TGF-β, activin, inhibin, leukemic inhibitory factor, oncostatin M, MIP-1-α, MIP-1β; MIP-2-α, GRO-α; MIP-2-β, platelet factor-4, macrophage chemotactic and activating factor and IP-10.

The fusion protein may be a secretory protein, which binds to a specific antibody of human albumin, and optionally, binds to a specific antibody of the CPSF in this fusion protein.

In another aspect of the invention, a recombinant vector is provided that comprises the sequence of the polynucleotide described above. The recombinant vectors

can be an expression vector for expressing the fusion protein encoded by the polynucleotide, HSA-CPSF, HSA-L-CPSF, or CPSF-L-HSA in a host organism. The host organism includes, but is not limited to, mammalian (e.g., human, monkey, mouse, rabbit, etc.), fish, insect, plant, yeast, and bacterium.

In a preferred embodiment, the host organism belongs to a genus of yeast such as Saccharomyces (e.g., S. cerevisiae), Pichia, Kluyveromyces, Torulaspora, and Schinosaccharomyces. In a more preferred embodiment, the host organism is Pichia pastoris. In a particular embodiment, the recombinant vector is a pPICZ A, pPICZ B, or pPICZ C.

Depending upon the host organism employed in a recombinant process for producing the fusion proteins, the recombinant fusion proteins of the present invention may be glycosylated or may be non-glycosylated. Preferably, when expressed in a host organism, the fusion protein of HSA and CPSF is glycosylated to substantially the same extent as that when expressed in mammalian cells such as Chinese hamster ovarian (CHO) cells, or as that when expressed in Pichia pastoris.

In yet another aspect of the invention, a recombinant cell is provided that is capable of expressing the sequence of the polynucleotide described above. The recombinant cell may constitutively or be induced in the presence or absence of an agent to express the fusion protein encoded by the nucleic acid, HSA-CPSF, HSA-L-CPSF, or CPSF-L-HSA in a host organism. The type of the recombinant cell includes, but is not limited to, mammalian (e.g., human, monkey, mouse, rabbit, etc.), fish, insect, plant, yeast, and bacterial cells.

When stored at ambient temperature or a lower temperature, the fusion protein of HSA and CPSF may have a shelf-life 2 times longer, preferably 4 times longer, more preferably 6 times, and most preferably 10 times, longer than that of the CPSF alone (i.e., not fused to HSA) stored under the same condition.

The fusion protein of the present invention may be administered to a mammal, preferably a human, via a variety of routes, including but not limited to, orally, parenterally, intraperitoneally, intravenously, intraarterially, topically, transdermally, sublingually, intramuscularly, rectally, transbuccally, intranasally, liposomally, via inhalation, vaginally, intraoccularly, via local delivery (for example by catheter or stent),

subcutaneously, intraadiposally, intraarticularly, or intrathecally. The antibody may also be delivered to the host locally (e.g., via stents or cathetors) and/or in a timed-release manner. In a particular embodiment, the fusion protein is delivered parenterally via injection.

When delivered in vivo to an animal, the fusion protein of HSA and CPSF may have a plasma half-life 2 times longer, preferably 4 times longer, more preferably 6 times, and most preferably 10 times, longer than that of the CPSF alone (i.e., not fused to HSA).

The HSA/CPSF fusion proteins of the present invention may also be administered in combination with a natural or recombinant human albumin, preferably a recombinant human serum albumin at a therapeutically effective dose and ratio.

In yet another aspect of the invention, combinations of different HSA/CPSF fusion proteins of are provided. The specific combinations of these fusion proteins may be administered to a patient to stimulate proliferation of multiple types of cells in the body or to synergistically enhance proliferation of a particular cell type.

In one embodiment, HSA/IL-11 fusion may be combined with HSA/EPO fusion and the resulting combination may be administered to a patient with a hematological disorder to simultaneously stimulate proliferation of erythrocytes and platelets.

In another embodiment, HSA/IL-3 fusion may be combined with HSA/EPO fusion and the resulting combination may be administered to a patient with a hematological disorder to enhance EPO-induced production of erythrocytes.

In yet another embodiment, HSA/IL-3 fusion may be combined with HSA/GCSF fusion and the resulting combination may be administered to a patient with a hematological disorder to increase the production of erythrocytes and neutrophiles, as well as eosinophils.

Alternatively, an HSA/CPSF fusion may be co-administered with a different HSA/CPSF fusion simultaneously or sequentially to a patient in need thereof. This combination therapy may confer synergistic therapeutic effects on the patients.

In yet another aspect of the invention, a method is provided for treating a patient with a CPSF in need thereof. In one embodiment, the method comprises: administering a

pharmaceutical formulation comprising a fusion protein of HSA and CPSF to the patient in a therapeutically effective amount. The pharmaceutical formulation may contain any pharmaceutically acceptable excipient and agents that stabilizes the HSA/CPSF fusion protein. The pharmaceutical formulation may further comprise natural or recombinant human serum albumin and/or another, different HSA/CPSF fusion protein.

The pharmaceutical formulation may contain any pharmaceutically acceptable excipient and agents that stabilizes the HSA/CPSF fusion protein. The pharmaceutical formulation may further comprise natural or recombinant human serum albumin and/or another, different HSA/CPSF fusion protein.

In another embodiment, the method comprises: administering a first pharmaceutical formulation comprising a first fusion protein of HSA and a first CPSF to the patient in a therapeutically effective amount; and administering to the patient a second pharmaceutical formulation comprising a second fusion protein of HSA and a second CPSF to the patient in a therapeutically effective amount. Such a combination therapy may confer synergistic therapeutic effects on the patient.

For example, HSA-IL-11 fusion protein may be administered to the patient first, followed by administration of HSA-EPO, HSA-GCSF and/or HSA-GMCSF at therapeutically effective doses and ratios to stimulate proliferation of different types of blood cells.

In yet another aspect of the invention, a kit is provided, comprising: a first fusion protein of HSA and a first CPSF, and a second fusion protein of HSA and a second CPSF. The first and second CPSFs may be the same or different. For example, the first CPSF is IL-11 and the second CPSF is EPO; the first CPSF is IL-3 and the second CPSF is EPO; or the first CPSF is IL-11 and the second CPSF is GCSF.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows nucleotide and amino acid sequences of embodiments of HSA-CPSF fusion proteins, HSA, and examples of individual CPSFs.

Figure 2 illustrates a plasmid DNA vector contains the HSA sequence and as a backbone vector for making HSA-CPSF fusion proteins.

Figure 3 shows a Western blot detected using mouse monoclonal anti-human serum albumin (Sigma Cat# A6684). Each lane was load with equivalent of 100ng of proteins. A), HSA (65Kd) from blood plasma; B), HSA (65Kd) from yeast; C), HSA/hG-CSF (84.2Kd); D), HSA/hEPO(83.5Kd); E), HSA/hIL-11(84.5Kd).

Figure 4 shows a Western blot detected using goat polyclonal anti-hIL-11 antibody (R&D Systems, Cat# Ab-218-NA), each lane contains 100ng proteins. A), human IL-11 expressed by *E. coli*; B), HSA/hIL-11 fusion protein expressed by yeast.

Figure 5 shows a Western blot detected using monoclonal anti-hGMCSF antibody (R&D Systems), each lane contains 20ng proteins. A), Human GMCSF expressed by *E. coli*; B), HSA/hGMCSF fusion protein expressed in yeast.

Figure 6 is a bioassay for human IL-11 and HSA/hIL-11 fusion protein in stimulation of T1165 cell proliferation. A), with hIL-6 antibody in medium; B), without hIL-6 antibody in medium.

Figure 7 is an ELISA bioassay for EPO and HSA fusion protein, HSA/hEPO.

Figure 8 shows the results of a stability test of rHSA/hIL-11 fusion protein under different temperature and its cell proliferation activity. A), 37°C; B), 50°C.

Figure 9 shows the results of an *in vivo* animal test of synergistic effects of a combination of different HSA-CPSFs in stimulating multiple blood cell proliferation, as compared with those when single HSA-CPSFs or CPSFs were administered.

Figure 10 shows a SDS-PAGE of purified HSA-CPSFs. Each lane was loaded with about 20μg of protein. A), HSA (65Kd) from blood plasma; B), HSA (65Kd) from yeast; C), HSA/hG-CSF (84.2Kd); D), HSA/hEPO(83.5Kd); E), HSA/hIL-11(84.5Kd).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides innovative compositions, kits and methods for modulating cell proliferation in vivo and more particularly for promoting cell growth for enhancing general health or for treating diseases or undesirable conditions.

In general, recombinant fusion proteins of human serum albumin (HSA) and a cell proliferation stimulatory factor (CPSF) are provided in order to circumvent problems associated with conventional therapy using the CPSF protein itself. Generally, compared with the CPSF protein alone, the inventive fusion proteins of the present invention possess the following advantages: 1) being capable of stimulating proliferation of multiple cell types, especially cells of various developmental lineages in the blood; 2) allowing a slower release of the HSA-CPSF fusion in the body to maximize the therapeutic effects of the CPSF, and/or 3) reducing potential side effects or toxicity associated with administration of CPSF alone.

The present invention also provides a method for treating a patient with a CPSF in need thereof. In one embodiment, the method comprises: administering a pharmaceutical formulation comprising a fusion protein of HSA and CPSF to the patient in a therapeutically effective amount. The pharmaceutical formulation may contain any pharmaceutically acceptable excipient and agents that stabilizes the HSA/CPSF fusion protein. The pharmaceutical formulation may further comprise natural or recombinant human serum albumin and/or another, different HSA/CPSF fusion protein. The pharmaceutical formulation may contain any pharmaceutically acceptable excipient and agents that stabilizes the HSA/CPSF fusion protein. The pharmaceutical formulation may further comprise natural or recombinant human serum albumin and/or another, different HSA/CPSF fusion protein.

In addition, the present invention also provides manufacturing processes efficient, cost-effective production for producing these recombinant fusion proteins in yeast. In particular, fusion proteins of HSA with each of human IL-11, EPO, G-GCSF and GM-CSF have been expressed in a yeast strain of Pichia pastoria and shown to have superior stability in storage and in plasma, and when combined, to possess synergistic effects on the production and growth of multiple types of blood cells.

1. HSA/CPSF Fusion Proteins

In one aspect of the invention, isolated polynucleotides are provided that encode fusion proteins formed between HSA and a CPSF, i.e., HSA/CPSF fusion. It should be noted other types of albumin can also be employed to produce a fusion protein with a CPSF of the present invention.

The CPSF may include any protein that can stimulate cell proliferation and/or production. In a particular embodiment, the CPSF is a hematopoietically active cytokine. Examples of such a CPSF are described in Aggarwal and Puri (1995) "Role of cytokines in immuno-regulation", in "Human Cytokines: Their role in disease and therapy", edited by Aggarwal and Puri, Blackwell Science Inc., Cambridge, Massachusetts, USA, pp28, which is incorporated herein by reference in its entirety.

Specific examples of the CPSF include, but are not limited to, colony-stimulating factors such as G-CSF, GM-CSF, eosinophil (EOS)-CSF (i.e. Interleukin-5), macrophage (M)-CSF (CSF-1), multi-CSF(i.e. IL-3) and erythropoietin (EPO); interleukins such as IL-1; IL-2; IL-4; IL-6; IL-7; IL-9; IL-10; IL-11; IL-12; IL-13 and IL-18; Steel factors (SLF: c-kit ligand; Stem-cell factor (SCF); mast cell growth factor); erythroid potentiating activity (EPA), Lactoferrin (LF), H-subunit ferritin (i.e., acidic isoferritin), prostaglandin (PG) E1 and E2, tumor necrosis factor (TNF)-α, -β (i.e. lymphotoxin), interferon (IFN) –α(1b, 2a, and 2b), -β, -ω and -γ, transforming growth factor (TGF)-β, activin, inhibin, leukemic inhibitory factor, oncostatin M; and chemokines such as macrophage inflammatory protein (MIP)-1-α (i.e. Stem-cell inhibitor); macrophage inflammatory protein (MIP)-1β; macrophage inflammatory protein (MIP)-2-α (i.e., GRO-β); GRO-α; MIP-2-β (i.e., GRO-γ); platelet factor-4; IL-8; macrophage chemotactic and activating factor and IP-10.

Four distinct colony-stimulating factors (CSFs) that promote survival proliferation and differentiation of bone marrow precursor cells have been well characterized: GM-CSF, G-CSF, M-CSF and Interleukin-3 (IL-3, Multi-CSF). Both GM-CSF and IL-3 are multipotent growth factors, stimulating proliferation of progenitor cells from more than

one hematopoietic lineage. In contrast, G-CSF and M-CSF are lineage-restricted hematopoietic growth factors, stimulating the final mitotic divisions and the terminal cellular maturation of partially differentiated hematopoietic progenitors. Erythropoietin is a hematopoietic growth factor that stimulated red blood cell proliferation. Growth factors, such as Stem Cell Factor, all the interleukins, and all the interferon, all are CPSF.

The CPSF may be linked directly to the N-terminus or the C-terminus of HSA to form an HSA-CPSF fusion. Optionally, there is a peptide linker (L) linking HSA and CPSF together to form the fusion protein: HSA-L-CPSF, or CPSF-L-HSA. The length of peptide is preferably between 2-100 aa, more preferably between 5-50 aa, and most preferably between 14-30 aa. The peptide linker may be a flexible linker that minimizes steric hindrance imposed by the bulk HA protein on CPSF, such as a $(G_4S)_{3-4}$ linker. The linker addition may be good for CPSF binds to its receptor.

The fusion protein may be a secretory protein, which binds to a specific antibody of human albumin, and optionally, binds to a specific antibody of the CPSF in this fusion protein.

In one embodiment, an isolated polynucleotide is provided that encodes a human serum albumin-interleukin-11 fusion protein (HSA-IL-11). The polynucleotide comprises a nucleotide sequence at least 90% identical to SEQ ID NO. 1. Preferably, the polynucleotide comprises a nucleotide sequence at least 95% identical to SEQ ID NO. 1. Preferably, the polynucleotide encodes an amino acid sequence comprising SEQ ID NO. 2. [HSA-IL-11].

In one embodiment, an isolated polynucleotide is provided that encodes a human serum albumin-interleukin-3 fusion protein (HSA-IL-3). The polynucleotide comprises a nucleotide sequence at least 90% identical to SEQ ID NO. 3. Preferably, the polynucleotide comprises a nucleotide sequence at least 95% identical to SEQ ID NO. 3. Preferably, the polynucleotide encodes an amino acid sequence comprising SEQ ID NO. 4. [HSA-IL-3].

In another embodiment, an isolated polynucleotide is provided that encodes a human serum albumin-erythropoietin fusion protein (HSA-EPO). The polynucleotide comprises a nucleotide sequence at least 90% identical to SEQ ID NO. 5. Preferably, the polynucleotide comprises a nucleotide sequence at least 95% identical to SEQ ID NO. 5.

Preferably, the polynucleotide encodes an amino acid sequence comprising SEQ ID NO. 6 [HSA-EPO].

In yet another embodiment, an isolated polynucleotide is provided that encodes a human serum albumin-granulocyte colony stimulating factor fusion protein (HSA-GCSF). The polynucleotide comprises a nucleotide sequence at least 90% identical to SEQ ID NO. 7. Preferably, the polynucleotide comprises a nucleotide sequence at least 95% identical to SEQ ID NO. 7. Preferably, the polynucleotide encodes an amino acid sequence comprising SEQ ID NO. 8 [HSA-GCSF].

In yet another embodiment, an isolated polynucleotide is provided that encodes a human serum albumin-granulocyte macrophage colony stimulating factor fusion protein (HSA-GMCSF). The polynucleotide comprises a nucleotide sequence at least 90% identical to SEQ ID NO. 9. Preferably, the polynucleotide comprises a nucleotide sequence at least 95% identical to SEQ ID NO. 9. Preferably, the polynucleotide encodes an amino acid sequence comprising SEQ ID NO. 10 [HSA-GMCSF].

In yet another embodiment, an isolated polynucleotide is provided that encodes a human serum albumin-CPSF fusion protein (HSA-CPSF). The polynucleotide comprises a nucleotide sequence at least 90% identical to SEQ ID NO. 11. Preferably, the polynucleotide comprises a nucleotide sequence at least 95% identical to SEQ ID NO. 11. Preferably, the polynucleotide encodes an amino acid sequence comprising SEQ ID NO. 12. Optionally, the polynucleotide further comprises a nucleotide sequence at least 90% identical to SEQ ID NOs. 13, 15, 17, 19, or 21. Preferably, the polynucleotide further comprises a nucleotide sequence encoding an amino acid sequence comprising SEQ ID NOs. 14, 16, 18, 20, or 22.

According to the embodiment, the CPSF may be selected from the group consisting of G-CSF, GM-CSF, (EOS)-CSF, CSF-1, EPO, IL-1; IL-2, IL-3, IL-4; IL-6; IL-7; IL-8, IL-9; IL-10; IL-11; IL-12; IL-13, IL-18, SLF, SCF, mast cell growth factor, EPA, Lactoferrin, H-subunit ferritin, prostaglandin (PG) E1 and E2, TNF-α, TNF-β, IFN–α, IFN-β, IFN- ω, IFN -γ; TGF-β, activin, inhibin, leukemic inhibitory factor, oncostatin M, MIP-1-α, MIP-1β; MIP-2-α, GRO-α; MIP-2-β, platelet factor-4, macrophage chemotactic and activating factor and IP-10.

The above-described polynucleotide with a sequence having a certain degree of sequence identity, for example at least 95% "identical" to a reference nucleotide sequence encoding a HSA/CPSF fusion protein, is intended that the polynucleotide sequence is identical to the reference sequence except that the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence encoding the HSA/CPSF fusion protein. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular nucleic acid molecule is at least 90%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the polynucleotide sequence encoding a HSA/CPSF fusion protein can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711). Bestfit uses the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2:482-489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

When stored at ambient temperature or a lower temperature, the fusion protein of HSA and CPSF may have a shelf-life 2 times longer, preferably 4 times longer, more preferably 6 times, and most preferably 10 times, longer than that of the CPSF alone stored under the same condition.

The present invention involves the utilization of albumin as a vehicle to carry a therapeutic protein such as a CPSF that can be used in the treatment of certain diseases such as cancers, or people in need of an increased blood cell proliferation in order to increase the blood cell numbers. The fusion protein of the present invention may be administered to a mammal, preferably a human, via a variety of routes, including but not limited to, orally, parenterally, intraperitoneally, intravenously, intraarterially, topically, transdermally, sublingually, intramuscularly, rectally, transbuccally, intranasally, liposomally, via inhalation, vaginally, intraoccularly, via local delivery (for example by catheter or stent), subcutaneously, intraadiposally, intraarticularly, or intrathecally. The HSA-CPSF may also be delivered to the host locally (e.g., via stents or cathetors) and/or in a timed-release manner. In a particular embodiment, the fusion protein is delivered parenterally via injection.

When delivered in vivo to an animal, the fusion protein of HSA and CPSF may have a plasma half-life 2 times longer, preferably 4 times longer, more preferably 6 times, and most preferably 10 times, longer than that of the CPSF alone.

The HSA/CPSF fusion proteins of the present invention may also be administered in combination with a natural or recombinant human albumin, preferably a recombinant human serum albumin at a therapeutically effective dose and ratio.

It is believed that after fusion with albumin, the CPSF protein can have a longer shelf-life and plasma half-life, which allows cost-effective storage and transportation, as well as reduces amount and/or frequency of drug administration.

It is noted that the same strategy may be used to develop stable fusion proteins with anticancer functions. For example, tumor suppressors, such as p53, pRb, pRb2/p130, and KLF6 may be fused with HSA. Optionally, insulin and insulin-like growth factor may be fused with HSA for therapeutic treatment of diabetes. The gene of antibody from human or other sources with therapeutic function also can be fused with albumin for easy delivery and therapeutic purpose. Antigens against which vaccines are developed can also be fused to albumin for in vivo delivery and increasing the antigenicity of the antigens. Antibody can be fused to albumin and then delivered into blood system to induce an immune response to the invaded foreign antigens such as

bacteria, parasites and viruses. In addition anti-aging proteins may be fused with albumin and delivered to humans.

2. Expression of Fusion Proteins in Host Organisms

The polynucleotides encoding the inventive HSA/CPSF fusion proteins can be cloned by recombinant techniques into vectors which are introduced to host cells where the fusion proteins can be expressed.

Generally, host cells are genetically engineered (transduced or transformed or transfected) with the vectors of this invention which may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the polynucleotides encoding HSA/CPSF fusion proteins. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

According to the invention, a recombinant vector is provided that comprises the polynucleotide sequence encoding an HSA/CPSF fusion protein. The recombinant vectors can be an expression vector for expressing the fusion protein encoded by the nucleic acid, HSA-CPSF, HSA-L-CPSF, or CPSF-L-HSA in a host organism. The host organism includes, but is not limited to, mammalian (e.g., human, monkey, mouse, rabbit, etc.), fish, insect, plant, yeast, and bacterium.

Expression of the polynucleotide encoding an HSA/CPSF fusion protein is under the control of a suitable promoter. Suitable promoters which may be employed include, but are not limited to, adenoviral promoters, such as the adenoviral major late promoter; or heterologous promoters, such as the cytomegalovirus (CMV) promoter; the respiratory syncytial virus (RSV) promoter; inducible promoters, such as the MMT promoter, a tetracycline or tetracycline-like inducible promoter, the metallothionein promoter; heat shock promoters; the albumin promoter; the ApoAI promoter; human globin promoters; viral thymidine kinase promoters, such as the Herpes Simplex thymidine kinase promoter; retroviral LTRs (including the modified retroviral LTRs hereinabove

described); the β-actin promoter; and human growth hormone promoters. The promoter also may be the native promoter which controls the polynucleotide encoding an HSA/CPSF fusion protein.

Also according to the invention, a recombinant cell is provided that is capable of expressing comprises the polynucleotide sequence encoding an HSA/CPSF fusion protein. The recombinant cell may constitutively or be induced in the presence or absence of an agent to express the fusion protein encoded by the nucleic acid, HSA-CPSF, HSA-L-CPSF, or CPSF-L-HSA in a host organism. The type of the recombinant cell includes, but is not limited to, mammalian (e.g., human, monkey, mouse, rabbit, etc.), fish, insect, plant, yeast, and bacterial cell.

In a preferred embodiment, the host organism belongs to a genus of yeast such as Saccharomyces (e.g., S. cerevisiae), Pichia, Kluyveromyces, Torulaspora, and Schinosaccharomyces. In a more preferred embodiment, the host organism is Pichia pastoris. In a particular embodiment, the recombinant vector is a pPICZ A, pPICZ B, or pPICZ C.

Depending upon the host employed in a recombinant process for producing the fusion proteins, the fusion proteins of the present invention may be glycosylated or may be non-glycosylated. Preferably, when expressed in a host organism, the fusion protein of HSA and CPSF may be glycosylated to substantially the same extent as that when expressed in mammalian cells such as Chinese hamster ovarian (CHO) cells, or as that when expressed in Pichia pastoris.

As indicated above, the albumin fusion proteins of the present invention are substantially preferably proteomic and can therefore be generated by the techniques of genetic engineering. The preferred way to obtain these fusion proteins is by the culture of cells transformed, transfected, or infected by vectors expressing the fusion protein. In particular, expression vectors capable of transforming yeasts, especially of the genus *Pichia*, for the secretion of proteins will be used.

It is particularly advantageous to express the HSA/CPSF fusion protein in yeast. Such an expression system allows for production of high quantities of the fusion protein in a mature form, which is secreted into the culture medium, thus facilitating purification.

The development of yeast genetic engineering has been made possible the expression of heterologous genes and the secretion of their protein products from yeast. The advantages of protein secretion (export) of yeast are including but not limited to, high expression level, soluble protein, corrected folding, easy to scale-up and easy for purification.

The HSA/CPSF fusion protein can be secreted into the media of yeast via an albumin natural secretion signal. The polypeptide sequence of HSA fusion protein can be preceded by a signal sequence which serves to direct the proteins into the secretory pathway. In a preferred embodiment the prepro-sequence of human albumin is used to secret the fusion protein out of yeast cells into the culture medium. Other secret signal peptides, such as the native *Saccharomyces cerevisiae* α-factor secretion signal, can also be used to make fusion protein of the present invention.

Yeast-expressed HSA is soluble and appears to have the same disulfide linkages as the human-blood derived counterpart. If used as a pharmaceutical, which may be potentially used in gram amounts in humans, a recombinant HSA will require a close identity with the natural HSA product. Secreting the HSA/CPSF fusion protein into the growth media of yeast, which is via prepro-amino-terminal processing (no initiator methionine residue), also circumvents the problems associated with preparing yeast extracts, such as the resistance of yeast cells to lysis. In addition, the purity of the product can be increased obtained by placing the product in an environment in which 0.5-1.0% of total yeast proteins is included and the lacks toxic proteins that would contaminate the product.

In a preferred embodiment, a particular species of yeast *Pichia pastoris* is used the system for expressing HSA/CPSF fusions of the present invention. *Pichia pastoris* was developed into an expression system by scientists at Salk Institute Biotechnology/Industry Association (SIBA) and Phillips Petroleum for high-level expression of recombinant proteins. The techniques related to *Pichia* are taught in, for example, US Patent Nos: 4,683,293, 4,808,537, and 4,857,467.

There are some advantages of using yeast *Pichia pastoris* to expression of HSA and HSA fusion proteins than using other systems. *Pichia pastoris* is a species of yeast genus, *Pichia*. *Pichia* has many of advantages of higher eukaryotic expression systems

such as protein processing, protein folding, and posttranslational modification, while being as easy to manipulate as E. coli or *Saccharomyces cerevisiae*. It is faster, easier, and less expensive to use than other eukaryotic expression systems such as baculovirus or mammalian tissue culture, and generally gives higher expression levels. *Pichia* has an additional advantage which gives 10 to100-fold higher heterologous protein expression levels. Those features make *Pichia* very useful as a protein expression system.

Owing to the similarity between *Pichia* and *Saccharomyces*, many techniques developed for *Saccharomyces* may be applied to *Pichia*. These include: transformation by complementation, gene disruption, gene replacement. In addition, the genetic nomenclature used for Sac has been applied to *Pichia*. For example, histidinol dehydrogenase is encoded by the HIS4 gene in both Sac and *Pichia*. The *Pichia* as a methylotrophic yeast is capable of metabolizing methanol as its sole carbon source. The first step in the metabolism of methanol is oxidation of methanol to formaldehyde using molecular oxygen by the enzyme alcohol oxidase. In addition to formaldehyde, this reaction generates hydrogen peroxide. To avoid hydrogen peroxide toxicity, methanol metabolism takes place within a specialized cell organelle, called the peroxisome, which sequesters toxic by-products away from the rest of the cell. Alcohol oxidase has a poor affinity for O_2, and *Pichia* compensates by generating large amounts of this enzyme. The promoter regulating the production of alcohol oxidase is the one used to drive heterologous (HSA or HSA fused) protein expression in *Pichia*.

Compared with *Saccharomyces* cerevisiae, *Pichia* may have an advantage in the glycosylation of secreted proteins because it generally does not hyper-glycosylate. Both *Saccharomyces* and *Pichia* have a majority of N-linked glycosylation of the high-mannose type; however, the length of the oligosaccharide chains added post-translation ally to proteins in *Pichia* (average 8-14 mannose residues per side chain) is much shorter than those in *Saccharomyces* (50-150 mannose residues). Very little O-linked glycosylation has been observed in Pichia. In addition, *Saccharomyces* core oligosaccharide have terminal α–1,3 glycan linkages whereas Pichia does not. It is believed that the α–1,3 glycan linkages in glycosylated proteins produced from *Saccharomyces* are primarily responsible for the hyper-antigenic nature of those proteins

making them particularly unsuitable for therapeutic use. Although not yet proven, this is predicted to be less of a problem for glycoprotein generated in *Pichia*, because it may resemble the glycoprotein structure of higher eukaryotes. Protein expressed as a secreted form for correctly refolding and easy to purification of HSA and HSA fusion proteins. Watanabe, et al. (2001) "In vitro and in vivo properties of recombinant human serum albumin from *Pichia pastoris* purified by a method of short processing time", Pharm Res 2001 Dec:18(12):1775; and Kobayashi, K et al. (1998) "The development of recombinant human serum albumin" Ther Apher, Nov:2(4):257-62.

There are many expression systems available for expressing in *Pichia*, such as EasySelect™ Pichia Expression Kit from Invitrogen, Inc. On this vector, an AOX1 promoter is used to allow methanol-inducible high level expression in Pichia and a Zeocin™ resistance as selective market for the recombinants from the transformation. Promoters (transcription initiation region) are very important in expression of fusion proteins in this invention.

AOX1 gene promoter is a very strong promoter in yeast system, especially in Pichia. Two Alcohol Oxidase Proteins are coded in Pichia for alcohol oxidase---AOX1 and AOX2. The AOX1 gene is responsible for the vast majority of alcohol oxidase activity in the cell. Expression of the AOX1 gene is tightly regulated and induced by methanol to very high levels, typically ≥30% of the total soluble protein in cells grown with methanol as the carbon source. The AOX1 gene has been isolated and a plasmid-bone version of the AOX1 promoter is used to drive expression of the gene of interest encoding the desired heterologous protein (Ellis et al., 1985; Koutz et al., 1989; Tschopp et al., 1987a). While AOX2 is about 97% homologous to AOX1, growth on methanol is much slower than with AOX1. This slow growth on methanol allows isolation of Mut^s strains (aox1). Except AOX1 gene promoter, other promoters can also be used to driver HSA fusion gene in yeast. They are including the promoter from, but not limited to, PGK1, GAPDH, Gal1, Gal10, CYC1, PH05, TRP1, ADH1, or ADH2 gene.

The expression plasmid can also take the form of shuttle vectors between a bacterial host such as E. coli, DH5a from GIBCO/Life Science and yeast; the antibiotic Zeocin are used to be a marker for HSA carrier vector in all the examples.

The expression vector contains the polynucleotide of HSA or HSA fusion therapeutic protein are introduced into yeast according to the protocols described in the kit from Invitrogen Inc.. After selection of transformed yeast colonies, those cells expressing the HSA fusion protein of interest are inoculated into appropriate selective medium and then tested for their capacity to secrete the given fusion protein into the extracellular medium. The harvesting of the protein can be conducted during cell growth for continuous cultures, or at the end of the growth phase for batch cultures. The fusion proteins which are the subject of this invention are then further purified from the culture supernatant by methods which take into account the albumin purification methods and pharmacological activities.

It is noted that other expression systems may also be used to express rHSA and HSA/CPSF fusion proteins, including but not limited to E. coli, B. Subtitis, Saccharomyces, Kluyverornyces, Candida, Torulopsis, Torulaspora, Schizosaccharomyces, Citeromyces, Pachysolen, Debaromyces, Metschunikowia, Rhodosporidium, Leucosporidium, Botryoascus, Sporidiobolus, Endomycopsis, animals, plants, and insect cells.

3. Combination Therapy of HSA/CPSF Fusion Proteins

The present invention also provides combinations of different HSA/CPSF fusion proteins. The specific combinations of these fusion proteins may be administered to a patient to stimulate proliferation of multiple types of cells in the body or to synergistically enhance proliferation of a particular cell type. In particular, a combination of human albumin fusions with different hematopoietically active cytokines is used to effectively promoting proliferation of the multiple blood cells and platelets. By using a combination of HSA/CPSF fusion proteins targeting the signal transduction pathways of different types of blood cells, multiple blood functional cell production, such as platelets, erythrocytes and macrophages of white cells, can be increased after administration by just one injection.

In the present invention, the albumin's plasma transporter function and the therapeutic function of the CPSF are integrated into a fusion form. The presence of

albumin may confer a superior stability to the CPSF by resisting degradation by proteases in the blood circulation, thus significantly prolonging the plasma half life of the CPSF. Due to the masking effect of a bulky albumin, different CPSFs fused with albumin in the combination may impose less interference with the biological function(s) of each other than a combination of the "naked" CPSFs. Further, a CPSF fused with albumin may be slowly released in the system over an extensive period of time, thereby reducing the toxicity associated with injection of the CPSF alone in abnormally high concentrations in the body. Such a slow release mode of action of the fusion protein combination can significantly reduce the amount and/or frequency of injections of the CPSF, thereby further reducing the side effects of CPSFs. Such combinations are particularly useful for stimulating multiple blood cell proliferation after or before the chemo- or radiation therapy of cancer patients who are tolerance for frequent, high dose injection of CPSF are seriously compromised.

For example, in animal testing human TPO, stem cell factor (SCF) and IL-3 have shown very strong toxicities *in vivo*. Due to the severe toxicity in vivo, SCF has been approved by the FDA for use in vitro only. The limitation on SCF has prevented it from being developed into useful therapeutics in the clinic. The side effects of IL-3 are dose-dependent. At a dose higher than 5μg/Kg, side effects have been observed in patients treated with IL-3, including fever, rash, fatigue, diarrhea, rigor, musculoskeletal pain, chills, headache, conjunctivitis, edema, chest pain, dyspnea, decrease in platelet counts, increasing in basophilic counts, marrow fibrosis, and pulmonary edema. Eder, M et al. (1997) "IL-3 in the Clinic", Stem Cells, 15:327-333.

According to the present invention, HSA fusion protein with this type of CPSF may remove above limitations by slowly releasing the drug into the patient's system. In addition, such fusion proteins may be combined with a relatively higher amount of albumin to further reduce the impact resulted from directly injecting the drug into the blood which causes a strong, adverse reaction of the central nervous system.

It is also known that "naked" cytokines (i.e., cytokines not fused to another protein such as HSA) are quite unstable when stored and have a short plasma half-life. Clearly, a therapeutic protein with such a weak stability in vivo constitutes a major handicap. In effect, repeated injections of the product, which are costly and inconvenient

for patient, or an administration of product by perfusion, become necessary to attain an efficient concentration in plasma. Due to its extended plasma half and enhanced stability, the HSA/CPSF fusion proteins of the present invention and their combinations, e.g., HSA fusions with hIL-11, hEPO, hG-CSF and hGM-CSF, can be used to stimulate the production of multiple blood cells in plasma of humans.

In one embodiment, HSA/IL-11 fusion may be combined with HSA/EPO fusion and the resulting combination may be administered to a patient with a hematological disorder to simultaneously stimulate proliferation of erythrocytes and platelets. For example, cancer patients may be injected with a combination of HSA/IL-11 and HSA/EPO fusion proteins, before or after, chemotherapy treatment to avoid blood transfusion and to stimulate proliferation of erythrocytes and platelets.

In another embodiment, HSA/IL-3 fusion may be combined with HSA/EPO fusion and the resulting combination may be administered to a patient with a hematological disorder to enhance EPO-induced production of erythrocytes.

In yet another embodiment, HSA/IL-3 fusion may be combined with HSA/GCSF fusion and the resulting combination may be administered to a patient with a hematological disorder to increase the production of erythrocytes and neutrophiles, as well as eosinophils.

Alternatively, an HSA/CPSF fusion may be co-administered with a different HSA/CPSF fusion simultaneously or sequentially to a patient in need thereof. This combination therapy may confer synergistic therapeutic effects on the patients. In one embodiment, the method is provided, comprising: administering a first pharmaceutical formulation comprising a first fusion protein of HSA and a first CPSF to the patient in a therapeutically effective amount; and administering to the patient a second pharmaceutical formulation comprising a second fusion protein of HSA and a second CPSF to the patient in a therapeutically effective amount. Such a combination therapy may confer synergistic therapeutic effects on the patient.

For example, HSA-IL-11 fusion protein may be administered to the patient first, followed by administration of HSA-EPO, HSA-GCSF and/or HSA-GMCSF at therapeutically effective doses and ratios to stimulate proliferation of different types of blood cells.

The present invention further provides a kit for use in the combination therapy described above. The kit comprises: a first fusion protein of HSA and a first CPSF, and a second fusion protein of HSA and a second CPSF. The first and second CPSFs may be the same or different. For example, the first CPSF is IL-11 and the second CPSF is EPO; the first CPSF is IL-3 and the second CPSF is EPO; or the first CPSF is IL-11 and the second CPSF is GCSF.

The HSA/CPSF fusion proteins and their combinations thereof may be used to treat a wide variety of diseases, including but not limited to the hematological disorders such as hypochromia, hypochromic microcytic anemia, and anemia, platelet-less, HIV infection, cancer, renal failure, and tissue/organ transplantation. These fusion proteins are preferred not to contain non-human sequences that may elicit adverse immunogenicity in the patient.

EXAMPLES

1. General Molecular Cloning Techniques

The classic methods of molecular cloning including, DNA preparative extractions, agarose and polyacrylamide electrophoresis, plasmid DNA purification by column or from gel, DNA fragment ligations, and restriction digestion, are described in detail in Maniatis T. et al., "Molecular cloning, a Laboratory Manual", Cold Spring Harbor laboratory, Cold Spring Harbor, NY., 1982 and will not be reiterated here.

Polymerase Chain Reaction (PCR) used through out all the examples is described by Saiki, R. K. et al, Science 230:1350-1354, 1985 and is carried out on a DNA thermal cycler (Perkin Elmer) according to the manufacturer's specification. DNA sequencing was performed by using standard facilities and following the method developed by Sanger et al., Proc. Natl. Acad. Sci. USA, 74:5463-5467, 1977. Oligonucleotides were synthesized by commercial facilities.

Transformation of *E. coli* was done by using DH5α competent cells from GIBCO/BRL. Qiagen plasmid DNA purification columns were used in the purification of plasmid DNAs. The transformation of yeast was carried out by electroporation following the instruction provided by the manufacturer or according to the manual of EasySelect™ *Pichia* Expression Kit (Invitrogen Inc). All yeast stains used in the examples are members of the family of *Pichia*, and in particular, the strain of *Pichia pastoris* (supplied by Invitrogen).

2. Construction of a Backbone Vector Expressing Human Serum Albumin

A total RNA isolated from human fetal liver was used in a reverse transcription polymerase chain reaction (RT-PCR) to generate the polynucleotide encoding human serum albumin. Briefly, 5μg of RNA was reverse transcribed by adding a poly$(T)_{18+N}$ primer and the SuperScript ™ II RNase H$^-$ reverse transcriptase (GIBCO/BRL) to make

the complementary first strand of cDNA. The reaction was incubated at 45°C for 20 minutes, then at 55 °C for 40 minutes.

The primers for cloning human serum albumin (HSA) are the following:

SEQ ID No. 23: 5'-GAATTCATGAAGTGGGTAACCTTTATTTCC-3' and
SEQ ID No. 24: 5'-GAATTCTTATAAGCCTAAGGCAGCTTGACTTGC-3'.

These primers were designed based on the HSA sequence published by GenBank (Access# V00494). Two EcoR I (underline of primers) sites were created at the 5' end and 3' end for sub-cloning into an expression vector. After inactivating the reverse transcriptase at 94 °C for 4 minutes, the DNA encoding of HSA was further amplified by Taq DNA PCR (Perkin Elmer) with 35 cycles of 94 °C/30 seconds and 58 °C/30 seconds and 72 °C/2 minutes 30 second, followed by a 72°C/10 minutes incubation. The PCR product (1842 base pairs) was confirmed by 1% agarose gel electrophoresis. The product was subcloned into a pCR II TA cloning vector from Invitrogen. DNA sequencing confirmed that the plasmid DNA contained an insert whose polynucleotide sequence matches the DNA sequence published in GenBank (Access# V00494). **Figure 1**, Seq ID No.11 is a polynucleotide DNA sequence and Seq ID No 12 is the protein amino acid sequence of human serum albumin.

After restriction digestion of the PCR product with EcoR I, the gel purified HSA DNA fragment was inserted into a pPICZ-A vector (provided by Invitrogen) at the EcoR I site. After transformation of bacteria DH5α cells with this vector encoding HSA, a colony was selected from a low salt LB-agar plate contains 25μg/ml Zeocin. The direction of the insert was confirmed by restriction enzyme double digestion of plasmid DNA by Xho I/Nde I. The construct was designated as pYZ-HSA (Y: yeast vector; Z: Zeocin resistant) and its physical map is shown in **Figure 2**.

There are some advantages associated with the vector constructed above. It confers resistance to the antibiotic Zeocin. Zeocin is isolated from *Streptomyces* and is structurally related to bleomycin/phleomycin-type antibiotics. Antibiotics in the family of bleomycin/phleomycin are broad spectrum antibiotics that act as strong antibacterial

and anti-tumor drugs. They show strong toxicity against bacteria, fungi (including yeast), plants, and mammalian cells. However, Zeocin is not as toxic as bleomycin on fungi. A single antibiotic Zeocin could be used in selecting the recombinants in both bacteria and in yeast. Further, there are multiple cloning sites at the 3' end of HSA for conveniently subcloning a CPSF protein in frame to encode a HSA-CPSF. In addition, a *myc* epitope sequence and a polyhistidine tag can be fused to the C-terminal of the expressed fusion protein for easy detection and/or purification by using commercially available antibodies against myc or polyhistidine tags. This vector, as a backbone vector, was used in the construction of expression vectors for all the HSA fusion proteins described in the Example section.

3. Molecular Cloning of Human IL-11, EPO, G-CSF, and GM-CSF

3.1. Molecular Cloning Of Human IL-11 Gene

Human Il-11 was cloned from a total RNA preparation of human bone marrow-derived stromal cells by RT-PCR method described in Example 2. The oligonucleotide primers are

SEQ ID NO. 25: 5'-CATATGAACTGTGTTTGCCGCCTGGTCC-3'

SEQ ID NO. 26: 5'-GATATGTATGACACATTTAATTCCC-3'

A polynucleotide having1051 base pairs (bp) was amplified from RT-PCR reaction and subcloned into pCR II TA cloning vector from Invitrogen Inc. DNA sequencing confirmed the reading frame of human IL-11 and inclusion of a 448 bp 3'-end un-translation region of hIL-11. An *Nde* I restriction enzyme site was created at the 5' end. The ATG initiate start codon of hIL-11 was included in this site (underlined in SEQ ID NO. 25). The DNA sequence of hIL-11 (SEQ ID NO. 13) and its amino acid sequence (SEQ ID NO. 14) are shown in **Figure 1**

3.2. Molecular Cloning of Human Erythropoietin Gene

Human erythropoietin gene was obtained by RT-PCR from human fetal kidney mRNA from Clontech Laboratory. EPO specific primers were designed based on the sequence published by Lin, FK et al., "Cloning and expression of human erythropoietin gene", Proc. Natl. Acad. Sci. USA., 82(22):7580-7584 (1985). They are

SEQ ID NO. 27: 5'-GGATCCATGGGGGTGCACGAATGTCC-3', and
SEQ ID NO. 28: 5'-GAATTCTCATCTGTCCCCTGTCCTGC-3'.

The PCR product was a full length reading frame of EPO with two newly created restriction enzymes in each end, Bam HI at the 5'end and EcoR I at 3'end. The product was inserted into pCR II vector and sequence confirmed. The human EPO DNA sequence (SEQ ID NO. 15) and amino acid sequence (SEQ ID NO. 16) are showed in **Figure 1**.

3.3. *Molecular Cloning Of Human G-CSF*

Primers used to clone the human G-CSF gene from a cDNA library of human fetal liver tissues are

SEQ ID NO. 29: 5'-GGATCCATGGCTGGACCTGCCACCC-3', and
SEQ ID NO. 30: 5'-GAATTCTCAGGGCTGGGCAAGGTGGC-3'

These primers were designed based on Nagata, S et al., Molecular Cloning and Expression of cDNA for Human Granulocyte Colony-Stimulating Factor, Nature, 319:415-418, 1986. A Bam HI site at 5'end and an EcoR I site at 3'end of G-CSF were created. The PCR products were gel-purified and subcloned into pCR2.1 TA cloning vectors and DNA sequence was confirmed. The human G-CSF DNA sequence (SEQ ID NO. 17) and the amino acid sequence (SEQ ID NO. 18) are shown in **Figure 1**.

3.4. *Molecular Cloning Of Human GM-CSF*

Human GM-CSF was cloned from a total RNA sample prepared from human fetal liver based on Wong, GG et al., "Human GM-CSF: molecular cloning of the

complementary DNA and purification of the nature and recombinant proteins" Science, 228:810-815, 1985. The Primers were:

SEQ ID NO. 31: 5'-GGATCCATGTGGCTGCAGAGCCTGCTGC-3', and
SEQ ID NO. 32: 5'-GAATTCTCACTCCTGGACTGGCTCC-3'

The PCR products were gel-purified and inserted into pCR2.1 TA cloning vector and sequence confirmed. The human GM-CSF DNA sequence (SEQ ID NO. 19) and amino acid sequence (SEQ ID NO. 20) are shown in **Figure 1**.

4. In Frame Fusion of HSA With Human IL-11, EPO, G-CSF or GM-CSF

There is a Bsu36 I site at the C'-terminus of HSA. All of the CPSFs described in the Example section were fused into this site by PCR primer extension to generate a restriction enzyme site of Bsu36 I at the N-terminus of the CPSF DNA sequence. The CPSF DNA fragments were amplified by PCR and then subcloned into Bsu36 I and Xho I sites of pYZ-HSA vector which had been double digested with Bsu36 I and Xho I to linearize the plasmid DNA

4.1. *Construction of Vector Containing Hybrid Polynucleotide of HSA/hIL-11*

IL-11 gene was fused to HSA C'-terminus by using the following PCR primers:

SEQ ID NO. 33: 5'-CTGCCTTAGGCTTACCTGGGCCACCACCTGGCC-3'. (Human IL-11 mature protein sequence is underlined), and
SEQ ID NO. 34: 5'-TGTCGACTCACAGCCGAGTCTTCAGCAGC-3'.

A Sal I site (underlined in SEQ ID NO. 34) was created at the 3' end of hIL-11 gene because there is a Xho I site in the sequence of mature protein of IL-11. The Sal I site sequence is a cohesive sequence with Xho I site. After ligation, the Sal I and Xho I site were all gone.

The PCR products were digested with Bsu36I and Sal I, and the fragment was gel purified and inserted into pYZ-HSA between of Bsu36 I and Xho I sites to generate a new plasmid DNA, pYZ-HSA/hIL-11. The HSA-hIL-11 hybrid polynucleotide sequence (SEQ ID NO. 1) and its fusion protein amino acid sequence (SEQ ID NO. 2) are showed in **Figure 1**.

4.2. Construction of Vector Containing Hybrid Polynucleotide of HSA/EPO

To make a HSA-EPO fusion protein, the following primers were designed

SEQ ID NO. 35: 5'-CTGCCTTAGGCTTAATCTGTGACAGCCGAGTCC-3'
(human EPO mature protein sequence underlined), and
SEQ ID NO. 36: 5'-CACTCGAGTCATCTGTCCCCTGTCCTGC-3'
(Xho I site underlined)

and used to generate the modified human IL-11 DNA fragment. The PCR products were inserted between Bsu36I and Xho I sites of pYZ-HSA to generate a pYZ-HSA/hEPO. The HSA-EPO hybrid polynucleotide sequence (SEQ ID NO. 5) and its fusion protein amino acid sequence (SEQ ID NO. 6) are shown in **Figure 1**.

4.3. *Construction of Vector Containing Hybrid Polynucleotide of HSA/HG-CSF*

Human G-CSF gene was fused with HSA DNA sequence by using two primers:

SEQ ID NO. 37: 5'-CTGCCTTAGGCTTAACCCCCCTGGGCCCTGCCAGC-3'
(G-CSF mature protein sequence underlined), and
SEQ ID NO. 38: 5'-CTCGAGTCAGGGCTGGGCAAGGTGG-3'
(Xho I site at the 3'-teminus of G-CSF underlined).

The PCR products were gel purified and subcloned between Bsu36I and Xho I sites of pYZ-HSA to generate a pYZ-HSA/hG-CSF. The HSA-G-CSF hybrid polynucleotide sequence (SEQ ID NO. 7) and its amino acid sequence (SEQ ID NO. 8) are shown in **Figure 1**.

4.4. Construction of Vector Containing Hybrid Polynucleotide Of HSA/HGM-CSF

The following primers:

SEQ ID NO. 39: 5'-ACTCCTTAGGCTTAGCACCCGCCCGCTCGCCCAGC-3'
(GM-CSF mature protein sequence underlined), and
SEQ ID NO. 40: 5'-CTCGAGTCACTCCTGGACTGGCTCC-3'
(Xho I site underlined)

were used to modify GM-CSF DNA sequence in order to subclone it into pYZ-HSA vector. PCR products were gel purified and double digested with Bsu36 I and Xho I and inserted between *Bsu36* I and *Xho*I sites of pYZ-HSA to generate a pYZ-HSA/hGMCSF. The HSA/GM-CSF hybrid polynucleotide sequence (SEQ ID NO. 9) and its fusion protein amino acid sequence (SEQ ID NO. 10) are shown in **Figure 1**.

5. Transformation of Yeasts

A yeast *Pichia pastoris* strain, GS115, colony was inoculated into 5ml of YPD medium in a 50ml conical tube at 30 °C overnight with shaking at 250 rpm. 0.2ml of the culture was inoculated into 500ml of YPD medium continually shaking at 30 °C for further 2-3 hours or until the cell density reach to OD_{600}=1.3-1.5. The cells were collected by centrifuging. The cell pellet resuspend in 500ml of ice-cold sterile water in order to wash the cells. After two rounds water washing, the cells were resuspended in 20ml of ice-cold 1 M sorbitol to wash again. The cells finally suspended in 1ml of ice-cold 1M sorbitol. The plasmid DNA constructs from Example 2, pYZ-HSA and in Example 4, pYZ-HSA/IL-11, pYZ-HSA/hEPO, pYZ_HSA/hG-CSF, and pYZ-HSA/hGM-CSF was linearized by *Pme*I restriction enzyme digestion first.

Five μg of each linear plasmid DNA was used to transform 80 μl of the freshly made yeast cells in an ice-cold 0.2cm electroporation cuvette. The cells mixed with plasmid DNA were pulsed for 5-10 ms with field strength of 7500V/cm. After the pulse, 1 ml of ice-cold 1M sorbitol was immediately added into the cuvette and the content was

transferred to a sterile 15ml tube. The transformed cells were incubated in 30 °C without shaking for 2 hours then spread on pre-made YPD-agar plates with 100μg/ml Zeocin. The colonies were identified with the insert and the expression level by SDS-PAGE or western-blot with proper antibodies.

A consortium of 4 yeast strains produced above (collected referred to as YZ-HSA/CPSFs) of Pichia pastoris encoding HSA/IL-11, HSA/hEPO, HSA/hG-CSF, and HSA/hGM-CSF was deposited to the ATCC under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. The 4 yeast strains are separately designated as YZ-HSA/IL-11, YZ-HSA/hEPO, YZ-HSA/hG-CSF, and YZ-HSA/hGM-CSF, respectively, and their consortium is deposited at the ATCC under Patent Deposit Designation No: PTA-4607.

Different strains of Pichia, such as X-33, KM71 and a proteinase deficient strain SMD1168 were tested for the expression and secretory of recombinant proteins.

6. Secretion and Characterization of HSA-CPSF Fusion Proteins Expressed by *Pichia*

Several colonies from each transformation of the HSA-CPSF were cultured with Zeocin in the buffered minimal medium with glycerol overnight or until OD_{600}=2-6 at 30 °C and shaking at 300 rpm. The cultured cells were collected by centrifuge at 1500 rpm for 5 minutes. Resuspend the cells into buffered minimal medium without glycerol and cell densities was keep in OD_{600} = 1.0. 100% methanol was added into each flask to a final concentration at 0.5% every 24 hours to induce the protein expression. The culture medium was collected at different time points and the expression of each fusion protein was confirmed by SDS-PAGE and western blot. The results showed that human albumin and HSA-CPSF fusion protein were expressed and secreted into the medium.

Mouse monoclonal anti-human serum albumin (Sigma) was used for immunoblotting on a SDS-PAGE gel. A typical Western blot experiment was carried on by electrophoresis transfer the protein from SDS-PAG to a nylon or nitrocellulose filter and incubated with a specific antibody (as the "first antibody"). Then an anti-first antibody would add to binding on the first antibody (as the "second antibody"). The

second antibody was labeled with Fluorescence and the filter was exposed to an X-ray film. Protein molecular weight standard was used to determine the protein size. The results (**Figure 3**) showed that the expressed recombinant proteins, HSA, HSA-CPSF therapeutic fusion protein, had an expected molecular weight and also had the same antigen as that of HSA prepared from a human blood plasma (Sigma). Using monoclonal anti-hIL-11 specific antibody as first antibody, the HSA/hIL-11 fusion protein and human IL-11 (R&D System) had the same antigen and showed that the molar ratio of HSA to hIL-11 in the HAS/hIL-11 fusion protein is as expected (**Figure 4**). Using monoclonal anti-hGMCSF specific antibody (R&D System) as first antibody, the HSA-GMCSF fusion protein and human GMCSF (R&D System) had the same antigen and showed that the molar ratio of HSA to GMCSF in the HAS/GMCSF fusion protein is as expected (**Figure 5**).

7. Purification and Molecular Characterization of HSA-CPSF

The cell culture medium (supernatant) containing the secreted protein of HSA or HSA-CPSF fusion protein produced from the recombinant *Pichia* was collected, the salt concentration reduced, and the pH was adjusted to above 7.5. The concentrated sample was passed through an Affi-Gel Blue-gel (50-100mesh) (Bio-Rad). The albumin or albumin fusion protein was bound to the matrix and eluded by a gradient 1-5M NaCl. 75-85% pure albumin or albumin-CPSF can be recovered in this step. If further purification is necessary, a size exclusion chromatography is applied to give a 95-99% purity of proteins. The pyrogen was removed from the protein samples in order to meet the requirement for use in *in vivo* test. The Affi-Prep Polymyxin Support (BIO-Rad) column was used to remove endotoxin from the samples. The purified protein finally passed through 0.2μM filter to be sterilized and the protein concentration was measured by a standard method by using a Bio-Rad Protein Assay Kit.

8. Cell Proliferation Assay of Human IL-11

A murine plasmacytoma cell line T1165 which is an IL-6 depended cell line was used to carry out a bioassay for IL-11 according to Paul, SR et al., PNAS 87:7512-7516, 1990. The cells were maintained in RPMI medium supplemented with 10% heat-inactivated fetal calf serum, 2mM glutamine, penicillin (100U/ml) and streptomycin (100μg/ml) (Gibco/BRL), 50 μM 2-meracaptoethanol (sigma), and recombinant human IL-6 (4-10ng/ml or 20U/ml in final concentration) (Gibco/BRL). Bioassay is preformed by $1X10^4$ cells/well were placed into96-well tissue culture plate in 200ul of IL-6 free medium for 48 hr in 37 °C in the presence of multiple dilution of hIL-11 purified from Thioredoxin fusion protein in *E .coli* or from yeast purified HSA/IL-11 fusion protein. In the final 6 hours, the cells were pulse-labeled with 0.5mCi of [^{3}H]thymidine (1Ci=37GBq, from Dupont) per well. After incubation, the cells were collected on to a glass filter, washed and assayed on a Beckman scintillation counter, Neutralizing goat anti-human IL-6 antibody was included to abrogate the effect of IL-6 as control in the testing of purified protein samples. The control proteins including with or without HSA (from human blood preparation), rHSA expressed in yeast (*Pichia*) in the lab, Thioredoxin (Trx) was a peptide purified from the enterokinase digested Trx/IL-11 fusion protein. The results are shown in **Figure 6** (**A** and **B** panels). The HSA/hIL-11 bioactivities were not affected by the presence of antibody of human IL-6. HSA fusion protein has about 1/3 of cell proliferation activity compared with that human IL-11 alone in the same amount of protein. Since HSA has a molecular weight about 3 times higher than that of hIL-11, it can be inferred that HSA-hIL-11 fusion protein has the same bioactivity as that of human IL-11 alone.

9. Bioassay of EPO by ELISA

Enzyme-linked immunosorbent assay (ELISA) kit from R&D Systems was used for the quantitative determination of erythropoietin (EPO) concentration and bioactivities comparison with a commercial EPO sample. The EPO ELISA is based on the double-antibody sandwich method. Microplate wells, precoated with monoclonal (murine) antibody specific for human EPO were incubated with samples or standard. Erythropoietin binds to the immobilized antibody on the plate. After removing excess

protein sample or standard, wells were incubated with an anti-EPO polyclonal (rabbit) antibody conjugated to horseradish peroxidase. During the second incubation, the antibody-enzyme conjugate bound to the immobilized EPO. Excess conjugate was removed by washing. A chromogen was added into the wells and oxidized by the enzyme reaction to form a blue colored complex.

The reaction was stopped by the addition of acid, which turned the blue to yellow. The amount of color generated was directly proportional to the amount of conjugate bound to the EPO antibody complex, which, in turn, was directly proportional to the amount of EPO in the protein samples or standard. The absorbance of this complex was measured and a standard curve was generated by plotting absorbance versus the concentration of the EPO standards. The EPO concentration of the unknown sample was determined by comparing the optical density of the protein samples to the standard curve. The standards used in this assay were recombinant human EPO (with kit) calibrated against the Second International Reference Preparation (67/343), a urine-derived form of human erythropoietin. Human recombinant EPO expressed in CHO cells was used as a control to determine the rHSA/EPO bio-activity.

The results showed that the bioactivity of hEPO fused to HSA had 1/10 of activity compared with the standard (**Figure 7**). The size of HSA-EPO fusion protein molecule may be too large, which prevents the anti-EPO antibody from efficiently binding to the EPO molecule fused to HAS, thereby reducing the sensitivity of the detection in this bioassay.

10. Stability Testing of HSA-CPSF Fusion Proteins *In Vitro*

Using HSA/hIL-11 as an example, the stability of this HSA-CPSF fusion protein was tested at different time points at 37 °C and 50 °C. 5ng of human IL-11 from bacteria or 5 ng of rHSA/hIL-11 was put into 200 µl thin-well PCR tube with 200 µl of tissue culture medium RPM1 without fetal bovine serum and other components. The tubes were sealed and left in water both. Samples were taken out at different time points and immediately put into –80 °C for storage. After all of samples were collected, a cell proliferation test on T1165 cell line was carried out by incorporating ^{3}H-Thymidine into

newly synthesized DNA of proliferating cells. The control of the test was set up in the same way as that in the bioassay of human IL-11 (See Paul et al., 1990). As shown in **Figure 8**, after 5 weeks in 37 °C (Panel **A**), the bioactivity of HAS/ IL-11 still remained the same, but the "naked" human IL-11 lost almost all of its bioactivity after three weeks at 37 °C. At 50°C (Panel **B**), the "naked" human IL-11 lost its the bioactivity completely in 2 weeks. And the HAS/IL-11 fusion protein still retained at least half of its bioactivity. These results indicate that a CPSF fused to human albumin can have a longer storage time and more resistant to degradation in harsh environment such as high temperatures.

11. Synergistic Effects of Combination of HSA-CPSF Fusion Proteins in Stimulation of Multicell Proliferation

Rabbits (2.3-2.6 Kg) were injected with 200 μl of recombinant proteins prepared above at day 1, day 3 and day 6. Rabbit A was injected with a mixture of 150U/kg EPO (about 10 μg of protein) and 100 μg recombinant HSA (rHSA); Rabbit B with a mixture of 120 μg IL-11 and 100 μg rHSA; and Rabbit C with a mixture of 120 μg rHSA/hIL-11 fusion (equivalent of 50μg of pure bacteria-expressed IL-11), 27 μg rHSA/hEPO fusion (equivalent of 150 U of HSA-EPO determined by using the EPO-ELISA Kit, R&D System Inc.) and 50 μg rHSA. Rabbit D was injected with 120 μg of HSA-IL-11 and about 80 μg rHSA .

Blood samples were collected and blood red cells and platelet numbers were counted by a hemacytometer. The results are shown in **Figure 9** (panels **A**, **B**, and **C**). The cell counts on the starting day of the experiment was treated as the base line. After the treatment with the proteins, the cell counts were compared with those on the starting day and the changes were plotted in the graphs in **Figure 9**.

As shown in panel A of **Figure 9**, both EPO and HAS/EPO fusion protein stimulated the production of erythrocytes in rabbit A and C, respectively. However, in rabbit A injected with the naked EPO, the level of erythrocytes reached a peak around day 35 post first injection and then declined quickly to reach near a baseline level around day 55. In contrast, in rabbit C injected with HAS/EPO fusion protein, the level of

erythrocytes increased and reached a plateau around day 35 post first injection but remained high till the end of the experiment. These results demonstrated that HAS/EPO fusion protein has a much longer plasma half-life than the naked EPO and remains bioactive for a much longer time than the naked EPO in vivo. As also shown in this panel, IL-11 had little effect in stimulation of erythrocyte production in rabbit B.

As shown in panel B of **Figure 9**, both IL-11 and HAS/IL-11 fusion protein stimulated the production of platelets in rabbit B and C, respectively. However, in rabbit B injected with the naked IL-11, the level of platelets reached a peak around day 20 post first injection and then declined quickly to reach near a baseline level around day 43. In contrast, in rabbit C injected with HSA/IL11 fusion protein, the level of platelets increased and reached a plateau around day 40 post first injection but remained high till the end of the experiment. These results demonstrated that HSA/IL-11 fusion protein has a much longer plasma half-life than the naked IL-11 and remains bioactive for a much longer time than the naked IL-11 in vivo. As also shown in this panel, EPO had little effect in stimulation of platelet production in rabbit A.

Panel C in Figure 9 compares the effects of a combination of HSA/EPO and HSA/IL-11 with HSA/EPO and HSA/IL-11 individually in stimulation of the production of erythrocytes and platelets on day 35 post first injection. As shown in this panel, compared with individual HSA/EPO and HSA/IL-11, a combination of HSA/EPO and HSA/IL-11 had much stronger effects in stimulating the production of both erythrocytes and platelets. For example, the erythrocyte level in the rabbit C which was injected with 27 μg of HSA/EPO in combination 120 μg HSA/IL-11 with is higher than the total erythrocyte level combining that in rabbit A (injected with 10 μg HSA/EPO) and rabbit D (120 μg HSA/IL-11). Since the molar ratio of HSA to EPO in the HSA/EPO fusion is about 5:1 and the amount of HSA/EPO in rabbit C is only 2.7 folds of that in Rabbit A, it can be predicted that if the amount of HSA/EPO is increased 5 folds so that a equal mole of HSA/EPO in both the HSA/EPO + HSA/IL-11 combination and HSA/EPO alone is administered, the erythrocyte level in the animal should be even higher with the administration of the combination. Based on this analysis, it can be reasonably inferred

that a combination of HSA/EPO + HSA/IL-11 fusion proteins has a synergistic effect in stimulating multiple blood cell production.

12. Expression and Scale-Up of HSA-CPSF Fusion Protein by Fermentation

In this example, it is shown that expression and scale-up are much easier by using a *Pichia* system than other currently available systems. After *Pichia* recombinants were isolated, expression of both Mut+ and Mut^S recombinants was tested. This involved growing a small culture of each recombinant, inducing with methanol, and taking sample at different time points. For secretory expression, both the cell pellet and supernatant were analyzed from each time point. The samples were analyzed on SDS-PAGE gels by using both Coomassie staining and Western blot. Bioactivities of expressed samples were tested and the expression levels and purity were monitored in each step for production of HSA fusion proteins. **Figure 10** shows a SDS-PAGE of the purity of various HSA-CPSFs fusion proteins.